National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 1932

Fluorescein Solution

This Standard Reference Material (SRM) is intended for use in establishing a reference scale for fluorescence intensity based upon MESF (molecules of equivalent soluble fluorophore) units [1–3]. This SRM is certified for the concentration of fluorescein with a certified purity in a borate buffer solution. The MESF scale is established for a particular set of experimental conditions by measuring the fluorescence intensity of known amounts of this SRM under the specified set of conditions as described in the "Instructions for Use" section of this certificate.

Each unit of SRM 1932 consists of three sealed amber glass ampoules containing a solution that consists of fluorescein in an aqueous borate buffer. Approximately 2.0 mL of the solution is flame-sealed into each individual ampoule that has been pre-scored for easy opening.

Certified Value: The certified concentration of fluorescein given below is based on gravimetric preparation and analysis of purity by proton nuclear magnetic resonance spectroscopy that was independently verified by analysis of impurities. A summary of fluorescein purity determinations is provided in Table 3. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by NIST [4]. The mass fraction (purity) of the fluorescein material used to prepare this SRM is 97.55 % ± 0.64 %.

Fluorescein: $60.97\ \mu mol \cdot kg^{-1} \pm 0.40\ \mu mol \cdot kg^{-1}$

Reference Values: Table 1 gives reference values for the molar absorption coefficient of the SRM 1932 fluorescein solution corrected for purity and fluorescence bias. NIST reference values are noncertified values, which represent the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [4] and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Table 1. Molar Absorption Coefficient of SRM 1932 Fluorescein Solution at 22.4 °C ± 0.5 °C

Wavelength (nm)	Molar Absorption Coefficient ($kg \cdot cm^{-1} \cdot mol^{-1}$)	Uncertainty ($kg \cdot cm^{-1} \cdot mol^{-1}$)
488.0	8.50×10^4	0.07×10^4
490.0	8.70×10^4	0.07×10^4
490.5	8.71×10^4	0.07×10^4
491.0	8.70×10^4	0.07×10^4

Expiration of Certification: The certification of this SRM is valid until **31 December 2006**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given here (see "Instructions for Use"). The certification is valid only for unopened ampoules that have been stored in the dark between 2 °C to 6 °C.

The overall direction and coordination of technical measurements leading to certification were performed by G.W. Kramer of the NIST Analytical Chemistry Division.

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Production and certification of this SRM were performed by P.C. DeRose, A.K. Gaigalas, B. Coxon, T.W. Vetter, and G.W. Kramer of the NIST Chemical Science and Technology Laboratory, with assistance from D.L. Duewer, J.B. Smeller, M.B. Satterfield, D.M. Bunk, M.J. Welch, E. White V, Y. Tewari, S.A. Margolis, J.R. Sieber, L.L. Yu, and L. Wang of the NIST Chemical Science and Technology Laboratory.

Ancillary measurements were provided by G.E. Marti, F. Abbasi, and J. Weaver of the U.S. Federal Drug Administration, R.F. Vogt of the Center for Disease Control and Prevention, and Y-z. Zhang of Molecular Probes, Inc.

Statistical consultation was provided by J. Lu of the NIST Statistical Engineering Division.

Packaging and ampouling of SRM 1932 were coordinated by M.P. Cronise of the NIST Standard Reference Materials Program.

The support aspects involved in the issuance of this SRM were coordinated through the NIST Standard Reference Materials Program by B.S. MacDonald of the NIST Measurement Services Division.

Information Values: Table 2 gives information values for SRM 1932. A NIST information value is a value that may be of use to the SRM user, but insufficient information is available to assess adequately the uncertainty associated with the value.

Table 2. Information Values for SRM 1932 Fluorescein Solution

pH (25 °C)	Buffer Concentration	Mass Density (22 °C)
9.48	0.10 $mol \cdot L^{-1}$	1.003 $g \cdot mL^{-1}$

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

Source of Material: The fluorescein (2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid, $C_{20}H_{12}O_5$, relative molecular mass 332.311, CAS No. 2321-07-5) powder used in SRM 1932 was prepared especially for this purpose by Molecular Probes, Inc.[1] (Eugene, OR, U.S.; Leiden, The Netherlands), 71358, MPR, Lot W018073. Boric acid granules were obtained from Mallinckrodt[1] (St. Louis, MO, U.S.), Lot 2549 KVTK, relative molecular mass 61.83. Sodium hydroxide pellets were from Mallinckrodt[1], Catalog No. 7708, Lot 7708M484721, relative molecular mass 40.00. All water used was NIST deionized water that was then subsequently passed through a second purification system (Millipore Mill-Q A10[1]) to produce water having a resistivity ≥ 18 MΩ cm.

[1]Certain commercial organizations, services, equipment, or materials are identified in this certificate to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the organizations, services, materials or equipment identified are necessarily the best available for the purpose.

Determination of Fluorescein Purity: Table 3 summarizes the purity determinations of the fluorescein material used to prepare SRM 1932.

Table 3. Summary of Fluorescein Purity Determinations and Associated Uncertainties

Constituent	Technique	Mass Fraction (%)	Uncertainty (%)
Fluorescein	^{1}H NMR[†]	97.55	0.64
DHBBA[*]	^{1}H NMR[†] & HPLC[‡]	1.04	0.20
Ethanol	^{1}H NMR[†]	0.21	0.04
Methyl Isobutyl Ketone	^{1}H NMR[†]	0.02	0.02
HOAc/Acetate	^{1}H NMR[†]	0.02	0.01
Ethyl Acetate	^{1}H NMR[†]	0.02	0.01
Water	Karl Fisher	0.25	0.02
Total Organics	CHO Analysis[#]	99.19	0.82
Potassium	FAES[¶]	0.50	0.14
Sodium	FAES[¶]	0.03	0.03
Chloride	Argentimetry[§]	0.37	0.03

[†] 500 MHz Proton Nuclear Magnetic Resonance Spectroscopy
[*] 2-(2',4'Dihydroxybenzoyl) benzoic acid
[‡] High Performance Liquid Chromatography and High Performance Liquid Chromatography-Mass Spectrometry, both normal and reversed phase
[#] Elemental analyses carried out by Schwartzkopf Microanalytical Laboratory, Inc.[1] (Woodside, NY, U.S.), Atlantic Microlab, Inc.[1] (Norcross, GA, U.S.), and Galbraith Laboratories, Inc.[1] (Knoxville, TN, U.S.)
[¶] Flame Atomic Emission Spectroscopy
[§] Halide content identified solely as chloride by X-ray Fluorescence and Inductively Coupled Plasma-Mass Spectrometry

Preparation of the SRM: The borate buffer solution was prepared by dissolving 18.56 g of boric acid granules in slightly less than 3 L of water. The pH was adjusted to above 9.1 using 40 mL of an approximately 3.0 mol•L^{-1} NaOH solution made by dissolving 29.87 g of sodium hydroxide pellets in 0.250 L of water. The mixture was diluted to 3.00 L with water to give a borate buffer with a boric acid concentration of about 0.10 mol•L^{-1}.

The SRM solution was prepared in a darkened room by dissolving 0.05636 g ± 0.00004 g of fluorescein (buoyancy corrected) in 2.7134 kg ± 0.0006 kg of borate buffer solution (buoyancy corrected). The mass of fluorescein used was corrected for the fluorescein purity yielding a final fluorescein concentration of 60.97 µmol•kg^{-1} ± 0.40 µmol•kg^{-1}. The fluorescein solution contained in an amber bottle was immediately aliquotted into ampoules that were subsequently flame-sealed. The pH and mass density of the SRM were determined from measurements on four ampoules selected randomly from the lot.

Assignment of Uncertainties: Standard uncertainty components equivalent to the estimated standard deviation were assigned for sample inhomogeniety and measurement uncertainties. These values were then combined with balance accuracy and estimated instrument method uncertainties using the root-sum-of-squares method. An expansion factor of $k = 2$ was applied so that the expanded uncertainties given in this certificate express an interval within which the true value is expected to fall with a level of confidence of approximately 95 % for a normal distribution [5].

SRM Stability: NIST has monitored the stability of a prototype fluorescein solution similar to SRM 1932 for over 24 months. Within the error of the measurements, the absorbance spectrum and the fluorescence spectral radiance (as indicated by the fluorescence signal spectrum) of the prototype solution did not change. Therefore, SRM 1932, if stored in the dark between 2 °C to 6 °C, is likely to maintain its original optical properties for the duration of its certification period. NIST will validate this conclusion by periodic monitoring of the stability over the lifetime of the SRM (see "Maintenance of SRM Certification" section).

INSTRUCTIONS FOR USE

CAUTION: This SRM is a solution contained in tip-sealed glass ampoules with pre-scored stems. Therefore, all appropriate safety precautions, including the use of gloves during handling, should be taken to avoid accidental breakage or spillage. Unopened ampoules should be stored in the dark between 2 °C to 6 °C in an upright position. The ampoules should **NOT** be frozen because of the possibility of breakage during freezing and thawing. Once the ampoule is opened, the solution should be used promptly with minimal exposure to any light (exposure to incandescent lighting is preferable to illumination from daylight or fluorescent lighting). Any unused solution in the ampoule should be discarded properly (see the "Material Safety Data Sheet" [MSDS] accompanying the SRM).

Opening an Ampoule: When an ampoule is opened, that area of the stem where the pre-scored band is located (around the gold band) should be wiped with a clean, damp cloth and the body of the ampoule wrapped in absorbent material. Then holding the ampoule steady and with the thumb and forefinger grasping the stem above the gold band, **minimal** thumb pressure should be applied to the stem to snap it. Correctly done, the stem should break easily where pre-scored. The use of a metal file to break the stem is **NOT** recommended.

The SRM 1932 solution should always be diluted gravimetrically at least one-hundred fold with the same buffer system used for the analyte of interest. Subsequent gravimetric dilutions can then be employed to generate a calibration curve. The calibration curve describes the relationship between fluorescence intensity, as determined by the fluorometer and the concentration of fluorescein, as determined by the gravimetric dilution. Care must be taken in making the gravimetric dilutions because uncertainties expand during this serial process. An error at one level adversely affects determinations at all subsequent levels. The calibration curve measurements should be generated starting with the lowest concentration. For best results, the conditions used for determining the calibration curve such as solution degassing, temperature, ionic strength, pH, etc., should closely match those used in the measurement of the analyte. Because fluorescence is a highly sensitive technique, great attention must be paid to the cleanliness of glassware and any other apparatus that contacts the solutions. Many plastics and gloves can contaminate samples with small amounts of highly fluorescent materials such as release agents and plasticizers. The running of blanks to check for such contamination is highly recommended.

Depending on the solution pH, aqueous fluorescein solutions are complex, rapidly equilibrating mixtures of its several forms (cation, neutral species, monoanion, and dianion). Each species has unique absorbance and fluorescence spectra. Above pH 9, aqueous fluorescein exists almost exclusively as the highly fluorescent dianion. However, as the pH of the solution is reduced, the concentration of the dianion decreases, and the concentrations of the much less fluorescent monoanion and neutral forms increase [6]. Accordingly, the sensitivity of the fluorometric assay for fluorescein also decreases, and quantitation becomes very dependent on knowing or maintaining the precise pH of the solutions during calibration as well during the assay itself. While the calibration strategy described above can work with solutions below pH 9, the uncertainties of such measurements inevitably grow larger as the solution pH is lowered.

REFERENCES

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[5] ISO; G*uide to the Expression of Uncertainty in Measurement*; ISBN 92-67-10188-9, 1st ed.; International Organization for Standardization: Geneva, Switzerland (1993); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://physics.nist.gov/Pubs/.

[6] Diehl, H.; *Studies on Fluorescein–VIII*; Talanta, Vol. 36 (7), pp. 799–802 (1989) and references therein; Sjöback, R.; Nygren, J.; Kubista, M.; *Absorption and Fluorescence Properties of Fluorescein*; Spectrochem. Acta, Part A, Vol. 51, pp. L7–L21 (1995).

Certificate Revision History: 01 December 2004 (This technical revision reflects updated absorption coefficient reference values (and related text) based on measurements corrected for bias associated with purity and fluorescence, rather than photobleaching; expiration date extended); 04 March 2003 (Original certificate date).

Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.